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Biodegradation of Hazardous Wastes

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Introduction

Biological treatment of hazardous wastes has the potential for effective, practical, and economical remediation of some Superfund sites and other hazardous waste problems. Some biological hazardous waste treatments may also be applicable for preventing the occurrence of future hazardous waste sites. From a public health perspective, this technology may allow for the treatment of environmental hazardous wastes such that potential human health effects are ameliorated, or indeed prevented, specifically by reducing the amount and toxicity of hazardous substances.

Considering the importance of the environmental concerns associated with hazardous wastes, specifically from a public health perspective, a conference on the "Biodegradation of Hazardous Wastes" was held April 9–10, 1990, at Utah State University. The main purpose of the meeting, and the focus of this report, was to provide an up-to-date review of some of the biological degradation research currently available and undergoing development and to provide a forum to discuss this technology's impact on human health and the environment. In addition, current and future use of biological treatment technologies were discussed, primarily from the perspective of technology transfer; and research needs and opportunities were identified. The meeting was chaired by S. Aust, Director of the Biotechnology Center at Utah State University, and the conference was organized by a committee consisting of Aust, A. Bourquin (ECOVA), J. Fouts (NIEHS), J. Loper (University of Cincinnati), J. Salanitro (Shell Development Company), W. Suk (NIEHS), and J. Tiedje (Michigan State University).

The conference brought together investigators in the fields of enzymology, microbiology, and molecular biology of hazardous-waste-degrading organisms, which included bacterial, fungal, and plant species. In addition, toxicological evaluation of biodegradation processes was discussed, essentially from the viewpoint of the agents under investigation, which consisted of the hydrocarbons, polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), volatile organics and chloro-organics, and benzene, toluene, ethyl benzene, and xylene (BTEX). Integrated within the context of the conference were presentations on the development of pertinent engineered systems and their applications in the field.

The conference documented the potential for biological degradation of hazardous wastes and clearly reflected the many recent advances and discoveries that have been made in this rapidly moving field. Several presentations documented the metabolism of chemicals previously regarded as resistant to biodegradation, for example, bacterial systems that oxidize trichloroethylene (TCE) or dehalogenate PCB. This was perhaps the overall theme of the meeting, for at one point biodegradation of hazardous organic chemicals was described in terms of a continuum in which easily degraded organics, such as BTEX, appeared on the left, and inordinately recalcitrant compounds, such as PCB, appeared on the right. On the left are those chemicals currently thought to be amenable to degradation, and on the right are those chemicals that have not been shown to be degraded. The consensus reached during discussion periods was that continuing discoveries and development of microorganisms, and new investigations into the mechanisms of biodegradation, are combining to reduce the number and type of hard-to-degrade chemicals. This in turn means that we can more easily degrade hazardous wastes. However, it was noted that biological treatability studies will require a complete understanding of the fate and transport of contaminants and the pathway used by the organisms to degrade the chemicals. Nonetheless, there was confidence that basic research would contribute significantly toward the development of this knowledge.

A panel discussion at the conclusion of the meeting noted the significant advances in the field and detailed future research needs, the strengths and weaknesses of current knowledge, and

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issues related to technology transfer. At the end of this meeting report, the reader should have an awareness of what is known and what is not, and what is required to span the breach. A summary of the meeting program is provided in the Appendix.

The presentations could be categorized by the chemicals being studied or otherwise addressed. The biodegradation studies could be classified as addressing petroleum hydrocarbons, chlorinated solvents, polycyclic aromatic hydrocarbons, azo dyes, and PCB. Other subjects, such as biological detoxification by means other than degradation, subjects of molecular biology of importance to biodegradation, new and unique biodegradation systems, etc., were also discussed at the conference. Finally, future research needs were addressed. This report is therefore divided into these topics.

Petroleum Hydrocarbons

Our current understanding of the genetics and physiology of the microbial degradation of alkanes and aromatic hydrocarbons was reviewed with regard to the potential for bioremediation and commercialization of biotechnology. B. Witholt (University of Groningen, the Netherlands) discussed microbial conversions of alkanes (e.g., C_6–C_{12} *n*-alkanes and alkenes) to alcohols, acids, epoxides, and poly-3-hydroxyalkanoate esters. The plasmid-borne alkane-degrading genes (alk-*BAC* operon) encoding for alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase, and electron-transfer proteins (rubredoxins) have been sequenced or partially characterized in *Pseudomonas oleovorans*. This bacterium, when using octane, produces up to 50% of its dry mass as poly-β-hydroxybutyrate polymer. Witholt observed that the chain length and degree of unsaturation of these biopolymers can be modulated by altering the carbon chain ratio of alkane/alkene in the culture medium. In addition, it may be possible to engineer multiphasic (20–80% hydrocarbon, water, and emulsion) fermentations that can produce significant quantities of alcohols, acids, epoxides, and biopolymers.

It is now recognized that bacterial gasoline degraders are ubiquitous in most soils. G. Hegeman (Indiana University) discussed the isolation of predominantly Pseudomonas species (54% *P. putida*), which can degrade petroleum alkanes and aromatic compounds. Organisms isolated used compounds according to three pattern types: toluene degraders (grow on toluene and benzene), xylene degraders (grow on xylenes), and octane degraders (grow on alkanes but not aromatics). Overlapping utilization patterns were rare among the strains isolated; however, those few isolated from contaminated sites could grow on alkanes and aromatics were resistant to toxic metals, and used TCE and isobutylbenzene (IBB). One isolate, a *P. stutzeri* strain, differed from known aromatic degraders *P. putida* F1 (uses toluene, phenol, and IBB) and *P. capacia* G4 (uses phenol) in that it grows on IBB but not toluene. This organism could cometabolize TCE when cultured on IBB or isopropylbenzene as a carbon source.

The diversity of soil bacteria able to use aromatic hydrocarbons was further illustrated by R. Olsen (University of Michigan) in comparing the ability of isolates from a contaminated aquifer to grow on benzene, toluene, ethylbenzene, or xylenes under aerobic (O_2 as terminal electron acceptor) or denitrifying (NO_3^-, O_2-free, gas-flushed) conditions. Most of the isolates (43 strains) such as Pseudomonas or Acinetobacter bacterial strains could be categorized into those that could grow aerobically on benzene, toluene, and ethylbenzene; benzene and toluene; toluene and ethylbenzene; or toluene alone. Under denitrifying conditions, the isolated strains could grow on benzene, toluene, and ethylbenzene, or toluene or ethylbenezene only; some cultures that grew on benzene, toluene, or ethylbenzene aerobically could not use these hydrocarbons under anoxic conditions in the presence of NO_3^-. The patterns of aromatic degradation were similar to those that have been described in the literature for *P. putida* strains PpF1 and PaW1, *P. mendocina* KR, *P. pickettii* PK01, and *P. cepacia* G4. A striking feature of a majority of these cultures is their inability to initiate growth on xylenes; very few could grow on *m*- and *p*-xylene, none grew on *o*-xylene. Subsequent experiments showed, however, that toluene can induce growth on xylenes and benzene. Olsen illustrated how toluene can be an inducer of four possibile metabolic oxidative pathways in which toluene is converted initially to either benzyl alcohol, *cis*-toluene dihydrodiol, *p*-cresol, or *o*-cresol. Induction of the second or fourth pathway would allow for growth on benzene for toluene-induced cells.

Studies by L. Young (New York University) also showed that microbes isolated from saturated soils are widely distributed with regard to their ability to degrade toluene under denitrifying conditions. Cultures were derived from enrichments containing 20 mM each of alkyl benzenes (benzene, toluene, and *o*-, *m*-, and *p*-xylenes) and 20 μM NO_3^-. One culture, T1, (gram-negative, nonfermentative, nonpseudomonad, rod-shaped bacterium) that metabolized toluene to CO_2 under denitrifying conditions was also able to cometabolize *o*-xylene when grown on toluene or pyruvate. T1 showed increased tolerance to toluene up to 2–3mM. Another enrichment culture growing on *m*-xylene was also shown to cometabolize benzene and *o*- and *p*-xylene.

Chlorinated Solvents

Short-chain chlorinated aliphatic hydrocarbons such as TCE are among the most persistent contaminants in groundwater. These compounds have been known to be the most prevalent compounds at Superfund sites and they are threats to human health because of their toxicity and carcinogenicity. When biodegradation does occur anaerobically, the carcinogen vinyl chloride is produced by reductive dechlorination. Several studies have revealed that TCE could be transformed aerobically by consortia of microorganisms (*1*), some soil microorganisms, including the ammonia-oxidizing bacterium *Nitrosomonas europaea* (*2*), and toluene- and phenol-oxidizing bacteria (*3*). Since these early studies, significant progress toward bioremediation technology has been made. This conference exemplifies that progress including a field demonstration of at least one technology (phenol-degrading G4) (*3*). The early discovery by Wilson and Wilson (*3*) that methanotrophs when grown on methane also oxidized TCE was dramatically proved by R. Hanson (University of Minnesota) when he showed that the broader substrate specificity of the soluble form of methane monooxygenase was most active toward TCE. Hanson examined several methanotrophs and other bacteria containing different types of oxygenases for their ability to oxidize TCE and other low-molecular-weight halogenated hydrocarbons. A comparison of bacteria that oxidize toluene, propane, methane, and naphthalene indicated that *Methylosinus trichosporium* OB3b

and *Methylosporovibrio methanica* 81Z oxidize TCE at rates much higher than other bacteria examined. Rates exceeding 10 g TCE oxidized/g cell dry weight per hour have been observed. These type II methanotrophs oxidized TCE and other halogenated hydrocarbons only when a soluble methane monooxygenase was expressed.

New developments in other organisms were presented by G. Hegeman (Indiana University) when he demonstrated the aerobic metabolism of TCE by several *Pseudomonas stutzeri* and *P. putida* strains isolated from hydrocarbon-containing landfarm soils. Standard soil enrichments with regular, unleaded gasoline containing TCE at concentrations of 1% and 1.11 mM, respectively, yielded several isolates of *P. stutzeri* that degraded TCE in the presence of isobutyl benzene. All strains that degraded TCE also degraded benzene, toluene, ethyl-, propyl-, and isobutyl-benzene. The presence of hexane and decane at 3 mM and 1.3 mM, respectively, blocked degradation of TCE and aromatic hydrocarbons in favor of alkane degradation in an artificial mixture of hydrocarbons simulating gasoline. Although the mechanisms of attack are still unknown, this work showed that there may be other significant mechanisms of attack that are yet unexplored and may prove useful for future technology development.

R. Unterman (Envirogen) discussed potential developments for use of the toluene-oxidizing system in the isolate *P. mendocina*. Envirogen recently received the right to use the engineered organism from Amgen and is planning field work in the near future. *P. mendocina* oxidized TCE via a monooxygenase system originally identified in the oxidation of toluene. Amgen scientists cloned the genes into *E. coli* and deregulated the organism for constitutive expression of the responsible enzyme. While the potential for this type system appears enormous, the application remains clouded due to regulatory problems concerning release of the organism into the environment.

The same toluene/phenol oxidizing system was originally characterized in an organism named G4 at the U.S. EPA laboratory in Gulf Breeze (*4*). Subsequently, ECOVA scientists have identified a proprietary compound that induces the organism to degrade TCE similar to phenol induction. M. Nelson (ECOVA) presented information on TCE degradation in a continuous-flow bioreactor with influent TCE concentrations of 1 - 5 ppm being degraded to below detectable levels (0.5 ppb). Activity of G4 in the reactor was maintained by addition of low levels of phenol. The results indicate the utility of the system for field applications using surface bioreactors in pump-and-treat processes. Subsequent laboratory studies identified conditions that would maintain TCE activity without the addition of phenol (on alternate inducing substrate) and thus be more suitable for use *in situ*. These conditions were tested in a bench-scale, simulated aquifer and resulted in removal of 96% of the TCE present. Using these conditions, ECOVA tested a pilot system in the field for developing and maintaining TCE-degradative activity within an aquifer. Initial concentrations ranged from 2.5–3 ppm TCE. After 24 hr of treatment, a down-gradient monitor-well had less than 0.5 ppm TCE; the concentration dropped below 0.1 ppm TCE after 7 days of operations. The test results indicated that *in situ* biological removal of TCE can be achieved in subsurface aquifers using G4 and the proprietary inducing substrate. This is probably the only field demonstration and it represents a significant advancement in biodegradation of these chlorinated solvent contaminants in groundwater. However, Nelson was not able to perform all the analyses necessary to satisfy the academic review of this work, a constant problem in client-driven field demonstrations. As well, none of the laboratory or field studies have addressed the problem of confirmed detoxification of contaminated environments. Some work has been initiated, at least at the laboratory level.

J. Ongerth (University of Washington) reported on the development of laboratory reactors for three heterogenous bacterial cultures, acclimated to the continuous presence of a consistent mixture of halogenated aliphatic and aromatic environmental contaminants. The three cultures were *a*) anaerobic, *b*) aerobic methanotrophic, and *c*) sequential anaerobic, aerobic-methanotrophic. The mixture of 20 chlorinated aliphatic and aromatic compounds was fed daily at 80 ppb to each reactor system. Significant levels of degradation were observed for more than half of the halo-organic compounds fed to each of the reactor systems. The sequential anaerobic, aerobic-methanotrophic culture reduced the concentration of all of the halo-organics to below detectable limits (generally 1 - 5 ppb).

The toxicity of the feed to and treated effluent from the anaerobic reactor system was examined using the Microtox bacterial assay. Values of EC_{50} ranged from 0.01 to 1.2 mmole for individual compounds, with a value of 0.05 mmole/*l* for the mixture. The low concentration of halo-organics in the reactor feed and effluent required concentration to enable use of the Microtox assay. Concentration of the halo-organics was accomplished by adsorption onto a synthetic polymer resin (XAD-2). Microtox assay EC_{50} values for reactor feed and effluent were normalized to the total organic halogen (TX) concentration. On this basis, the toxicity of reactor effluent concentrates averaged an EC_{50} of 0.123 mg TX/*l*. However, since the TX of anaerobic reactor effluent was about 15% of reactor influent, the absolute toxicity of the feed stream was being reduced through treatment.

Polycyclic Aromatic Hydrocarbons

R. Vestal (University of Cincinnati) reported on research that he and D. Warshawsky and colleagues have conducted at the University of Cincinnati. Their interest is directed to the microbial degradation of the recalcitrant, carcinogenic 4- and 5-ring PAHs and the carcinogenic *N*-heterocyclic aromatics. Although the microbial mineralization of 2- and 3-ring PAHs has been well described, few if any microbial systems have been developed for degradation of members of these classes of compounds. Also, this is a group of compounds for which the pathway and extent of biodegradation is likely to be important. It is not known whether microbes could produce stable intermediates that might pose more of a health hazard than the parent compounds. For example, mammalian metabolism is known to produce carcinogenic intermediates such as the *trans*-7, 8-dihydrodiol derivative of benzo[*a*]pyrene and the 3-hydroxy derivative of 7H-dibenzo (*c*,*g*) carbazole.

Environmental sites highly contaminated with PAHs and *N*-heterocyclics include the more than 1100 former gas and tar production plants in the United States. Gas for lighting and heating was manufactured from coal for more than 100 years before the 1960s. Today these manufacturing sites, generally surrounded by heavily populated areas, still present high concentrations of

residues in trapped pools of oil and tar or spread out over the surrounding soil as well as underground and surface waters. These sites also provide a likely source of microorganisms capable of metabolizing PAHs and *N*-heterocyclics.

The researchers used isotopically labeled pyrene, benzo-[*a*] pyrene (BaP), and carbazole to test the extent of their mineralization in soil suspensions from three abandoned coal gasification plants. Mineralization of each compound was observed during 7-week incubations. Subsequently, a discrete microorganism was isolated that degraded each of the compounds. A major finding was that in each case the mineralization by indigenous microbes can be significantly enhanced by the reintroduction of the isolated bacteria. For example, when an isolated pyrene degrader, grown on pyrene, was reintroduced into soil at 9×10^9 colony forming units/g, mineralization of pyrene was 55% within 2 days, compared to 1% for only the indigenous microbes. These highly encouraging results suggest that efficient microbial systems may become available for the removal through mineralization of carcinogenic PAHs and *N*-heterocyclics and their metabolites.

Azo Dyes

Degradation of azo dyes was featured in presentations by P. Bishop and W. Tabor, both of the University of Cincinnati. Azo dyes comprise 60% of all coloring agents in use today, resulting in the disposal of about 60,000 tons per year. Past practices in their widespread manufacture and use have resulted in high concentrations of azo dyes and related dye products in soil pits and lagoons, leading to contamination of waters, sediments, and bottom-feeding fish by these dye-related components. Today, various azo dye discharges are routed into municipal waste-treatment facilities, where some are biodegraded along with more conventional organic pollutants by microorganisms in the reactor. Others are not degraded during treatment but sorb to sludge or are discharged in the effluent stream.

The major significance of azo dyes to human health is in the carcinogenic potential of aromatic amines that arise as metabolic products upon reductive cleavage of the azo linkage. The objective of these researchers is to optimize aerobic microbial degradation of azo dyes, avoiding the potential for aromatic amine generation by destruction of the C–N linkage.

Aerobic, activated sludge from a mixed domestic/dye industry waste-treatment facility was used as the source of microorganisms. Bishop presented the case for fixed film bioreactors, using Acid Orange 7 (AO7) as a model azo dye known to be metabolized by activated sludge. Biofilm systems have solids retention times 10-fold longer than the 3–10 days typical of activated sludge systems. The films support different microbial populations that degrade different substrates at different levels in the biofilm. These features facilitate the adaptation and retention of specialized microbial systems.

Using a bench model rotating biofilm reactor, high AO7 removal rates were seen relative to those observed with an aerobic suspension reactor. Analyses of the effects of environment on removal kinetics in the biofilm will be used as a basis for studies on the degradation of more recalcitrant azo dyes.

Tabor and colleagues have emphasized microbial enrichment and product analysis in their research on oxidative degradation of azo dyes. Activated sludge cultures were supplemented with AO7, with Acid Red 151 (AR151), a structurally similar dye known to be much more refractory to microbial degradation, or with structurally related moiety component chemicals such as sulfanilic acid.

A consortium of microbes was obtained that was capable of degrading AO7 at about 10 mg/*l* in 7–10 hr at a titer of $10^6 - 10^8$ colony forming units/mL. Greater than 95% of AO7 was removed together with the transient production of low levels of sulfanilic acid. This degradation proceeded in the presence of high aeration, unlike the decolorization of AO7 that was observed by any of several *Pseudomonas sp* in incubation conducted under reduced oxygen conditions.

Development of microbial consortia and isolation of relevant microorganisms are being pursued in relation to AR151 and other more refractory dyes. In the process of studies on AR151, abiotic decolorization was observed to occur via a free-radical mechanism enhanced by metal ions such as Cu(II), Cr(VI), and Fe(III). It appears these complementary microbiological, analytical, and bioreactor engineering studies offer a fruitful approach to the oxidative degradation of azo dyes.

Polychlorinated Biphenyls

PCBs pose a substantial challenge for developing a biological cleanup technology. If one considers hazardous waste targets as a spectrum from the easiest to the most difficult to degrade (e.g., gasoline and dioxin), PCBs are at the difficult end. This complex family includes over 200 specific PCB congeners, most of which are difficult to biodegrade. Unlike many rapidly degraded chemicals, such as gasoline and 2, 4-D, the chlorinated biphenyls (as opposed to biphenyl) are not usable as a carbon and energy source. Thus, their biodegradation will require the development of cometabolic processes. In addition, they are highly insoluble and not readily bioavailable, an added challenge to be addressed.

R. Unterman (Envirogen) summarized work on aerobic PCB metabolism relevant to bioremediation and outlined the research directions important for practical application of PCB bioremediation. He reported that microbiological, biochemical, and genetic studies have demonstrated that at least two types of aerobic PCB-degrading strains exist, type I and type II. The biodegradative activity of these two types is complementary in that each can degrade specific congeners that the other has difficulty in degrading. Current studies are underway to assess the potential of using mixed cultures of native type I and type II strains to effect a greater extent of PCB degradation. Genetic studies are also underway to isolate the genes from these two types of organisms with the ultimate goal of developing a chimeric PCB-degrading strain expressing an even broader and more extensive degradative competence.

The application of both naturally occurring and genetically engineered strains will require scale-up and process development in collaboration with the engineering disciplines. Both *in situ* and reactor-based processes (soil slurry bioreactors) are envisaged with the ultimate goal of biotreating PCB-contaminated soils and sediments. To date, the direct aerobic approach for bioremediating PCB-contaminated soils is probably limited to Aroclors 1242 and 1248 at concentrations of 1000 ppm and lower. Further advances in the development of these aerobic strains should eventually allow degradation of even higher concentrations of PCB contamination.

Complementary to the aerobic studies outlined above, more recent research has now demonstrated the anaerobic reductive dechlorination of PCBs. These were summarized by D. Abramowicz (General Electric Corp.). This biotransformation sequentially removes the chlorine atoms from the biophenyl nucleus, thereby generating lower-chlorinated products from higher-chlorinated congeners. These lower-chlorinated PCBs are more susceptible to aerobic biodegradation. This suggests that it might be possible to develop a dual anaerobic/aerobic bioremediation processes whereby an initial dechlorination generates PCB products that are readily degraded by aerobic strains, even as a sole source of carbon and energy. The potential of this concept has been verified; Abramowicz reported enhanced PCB removal by a sequential anaerobic then aerobic incubation in the laboratory over that removed by each process separately.

Abramowicz reported that several laboratories have now clearly shown that microorganisms from PCB-contaminated sites in the Hudson River dechlorinate Aroclor 1242. His group has found that rates of dechlorination can be stimulated by the addition of trace metals to the medium and that the Hudson River is the best source yet found for microorganisms that degrade PCBs, removing nearly all of the *meta* and *para* chlorines. Studies with single PCB congeners have revealed pathways of dechlorination. These often do not show a single sequence of intermediates, but they do show predominate routes to lesser chlorinated products. A particularly interesting finding is that the pathways of dechlorination are not the same for all sediments. This finding, together with the results of the Michigan State University group who showed different positions dechlorinated in Aroclors 1242 and 1260 by two different sediments, suggests that different microbial populations, or at least some different biochemical features, direct the dechlorination toward different positions for the same PCB molecule. This also suggests that the dechlorination is not catalyzed by a simple chemical catalyst.

Practical schemes of PCB remediation by dechlorination would be greatly aided if one could selectively grow larger masses of PCB dechlorinators and thus enhance the rate of PCB transformation. J. Tiedje (Michigan State University) reported on the attempts of their group to enrich PCB dechlorinators using dechlorination as the electron sink or acceptor and using the information learned from the 3-chlorobenzoate dechlorinating strain, strain DCB-1, as a guide. These enrichments, now in their fourth transfer, have maintained but not enhanced their dechlorination rate. Tiedje reviewed the features of strain DCB-1 that may be relevant to dechlorination of PCBs or other chloroaromatics. First, DCB-1, like most aromatic dechlorinating populations studied so far, is fairly specific for chloroaromatic substrate that it can attack. It is specific for chlorine or bromine in the *meta* position of benzoates, although it also has some activity on tetrachloroethene. Because DCB-1 is a distinctive large rod, it could be recognized under the microscope if it were present in other dechlorinating enrichments. It is not. Thus, there must be other dechlorinating strains in other extant enrichments, including other chlorobenzoate enrichments. Second, DCB-1 has recently been shown to grow using dechlorination as its sole electron acceptor with H_2 or formate or its electron donor. Thus, dechlorination is an energy-yielding reaction, and there is a potential benefit to organisms that can dechlorinate any chlorinated substrate, including PCBs. Tiedje reported that organisms from the Hudson River upstream from the PCB contamination and organisms from a nonpolluted Michigan river did not dechlorinate PCBs within the time period that organisms from several PCB-contaminated sediments did. This suggests that there may be populations that were benefiting (by growth) from PCB as their electron acceptor. Third, DCB-1 is a novel bacterium with an unusual combination of features; perhaps some of these features are related to dechlorination. These features include mixotrophic growth, CO_2 fixation by the acetyl CoA synthetase pathway, thiosulfate disproportionation, N_2 fixation, and H_2 lithotrophy. The organism reduces sulfate to sulfide and belongs to the class of sulfate-reducing bacteria. However, none of a number of other sulfidogens related phylogenetically or with one or more of these properties dechlorinated chlorobenzoate. Thus, aromatic dechlorination appears to not be a property of most sulfidogens.

The PCB dechlorination reactions are of particular relevance to toxicology for two reasons. First, the *meta* and *para* chlorines are removed by reductive dechlorination. Removal of these chlorines from the coplanar PCBs should reduce dioxinlike toxicity. Tiedje reported that Aroclor 1242 augmented with 3, 4, 3′, 4′-PCB and 2,3,4,3′,4′-PCB showed nearly complete removal of these toxic congeners. Furthermore, his colleagues in analytical chemistry at Michigan State University (Enke and Lopshire) demonstrated 80–95% reduction in the coplanar PCBs in Aroclor 1242 after incubation with PCB-dechlorinating consortia. Thus, toxicity appears to be reduced by an order of magnitude by dechlorination. However, this selective dechlorination leaves *ortho*-enriched PCBs that are not present in commercial PCBs. The toxicology of these PCBs has not been evaluated. This is an excellent example of where microbiologists and toxicologists should interact to evaluate the unique intermediates produced by biotransformation.

While considerable progress has been made in demonstrating that most PCB congeners can be metabolized either anaerobically or aerobically, the next challenge, bioavailability, appears formidable. Abramowicz reported that their best aerobic, PCB-degrading strain removed only 25% of the PCB in a drag-strip soil contaminated some years ago with waste PCBs, while the same strain removed 70% of the PCBs when first coupled with Soxhlet extraction and 90% of the PCBs in the Aroclor standard. However, when anaerobic dechlorinating sediments (25%) were mixed with the drag-strip soil (75%), 50–90% of the higher chlorinated congeners were removed. The anaerobic dechlorinating systems may have an advantage in overcoming some of the bioavailability problems of PCBs.

Other Subjects

The immobilization of pollutants in soils by polymerization reactions or oxidative (covalent binding) coupling to humic substances as a means of detoxifying compounds was discussed by J.-M. Bollag (Pennsylvania State University). Soil microbial laccases, catechol oxidases, tyrosinases, and peroxidases are in part responsible for these fixation mechanisms with such compounds as naphthols, anilines, chlorinated phenols, and methoxyphenols. In cross-coupling reactions, these structures are converted to dimer, trimer, tetramer, and pentamer derivatives that link to humic acids. Bollag showed that 2, 4-dichlorophenol

(an intermediate in 2,4-D degradation) can be linked by soluble or immobilized laccase (polyphenol oxidase) to humic and fulvic acid derivatives, e.g., vanillin, syringic acid, or guaiacol. The co-polymerization of simazine, a triazine herbicide, and 4-chloroaniline was catalyzed by oxidoreductases with guaiacol to form *N*-heterocyclic trimers. In a study with 4-chloroaniline it was shown that horseradish peroxidase or laccase from the fungus *Trametes versicolor* can form oligomers of this compound by free-radical coupling oxidations. Bollag also presented evidence that chlorinated phenol compounds that polymerized onto humic and fulvic material were slowly released, suggesting that these natural or enhanced transformations may be used to immobilize or inactivate toxic compounds or their metabolites in soils and sediments.

The molecular biology of xenobiotic metabolism and the potential for the genetic selection and manipulation of microbes having enhanced biodegradative potential were discussed by A. M. Chakrabarty (University of Illinois Medical Center). It is now well established that genes encoding for the degradation of many chlorinated and nonchlorinated compounds (e.g., petroleum hydrocarbons, PCBs, 2,4,5-T, and 2, 4-D) are plasmid-borne in bacteria. Chakrabarty showed how the selection of strains that can degrade 4-chlorobenzoate and 3,5-dichlorobenzoate, chlorocatechol, 2, 4-D, 2,4,5-T, and *p*-chlorobiphenyl from species metabolizing unchlorinated analogs results in the evolution of completely new sets of enzyme activities with altered specificity and regulation. Experiments were described in which microbes from a waste site were grown in a chemostat containing strains of *P. putida* carrying degradative plasmids for toluic acid, salicylate, and 3-chlorobenzoate. This mixed culture was adapted on these compounds plus low levels of 2, 4, 5-T herbicide. After modulating these substrate concentrations for 11 months, an organism designated as *P. cepacia* AC1100 was isolated, which grew on 2, 4, 5-T as sole carbon and energy source. Strain AC1100 when inoculated into soil was able to degrade 5000 ppm 2, 4, 5-T to <10 ppm. Other soil experiments indicated, however, that low levels (2–10 ppm) of the herbicide are not degraded in inoculated soils because 2, 3, 5-T is irreversibly bound and unavailable for AC1100 growth or a breakdown intermediate may be toxic to bacteria. AC1100, however, declines to nondetectable concentrations (6 weeks) when its primary substrate, 2, 4, 5-T, decreases to levels that do not support growth and persistence. The inability to degrade low levels of xenobiotics may represent a technical difficulty with the use of microbial cultures for soil or hazardous waste inoculations.

The degradation of hazardous wastes by white rot fungi was discussed by S. Aust. These fungi mineralize a wide variety of chlorinated organics, pesticides, PCBs, dioxins, and PAH compounds. The biochemical characterization of the lignin peroxidases from the white-rot fungus, *Phanerochaete chrysosporium*, was described by M. Tien (Pennsylvania State University) and J. Bumpus (Utah State University). The heme-containing lignin peroxidase isoenzymes formed during growth in nitrogen-limiting medium include the lignin peroxidases and manganese-dependent enzymes. Absorption spectra studies confirmed that a heme-linked ionization of an imidazole (histidine) residue in the reduced form of the enzyme was similar to that described for plant peroxidases (e.g., horseradish and turnip peroxidases). In addition, electrochemical titrations of a predominant ligninase isozyme (H8) showed that the protein carries out a one-electron transfer probably via the formation of an oxyperoxidase in which ferric-enzyme is converted to a ferrous-enzyme state followed by reaction with O_2 or excess H_2O_2. Tien also discussed the isolation of a mutant strain of *P. chrysosnorium* that can grow in high *N*-containing media (nitrogen-deregulated) and produced 10–15 times the amount of lignin peroxidase, as do cultures of the native fungus. The persistence and growth of the fungus and its ability to degrade xenobiotics in soils inoculated with this new strain, however, has not been tested.

The potential for using green plants to degrade xenobiotic chemicals was discussed by M. Gordon (University of Washington, Seattle). Plants have the ability to detoxify compounds through *a*) conjugation to carbohydrates and amino acids, *b*) oxidation reactions, *c*) ring cleavage of catechols and hydroxybenzoates, *d*) deposition of tannins and pigments, and *e*) formation of condensed rings (quinones). Gordon indicated that plants can be genetically-engineered to carry and/or incorporate degradative plasmids or gene clusters using the Aarobacterium tumor transformation system or electroporation techniques to enhance plant cell protoplast fusion. It has been possible to clone the 2,4-dichlorophenol (2,4-DCP) hydroxylase from Alcaliaenes into Aarobacterium, which was then introduced into tobacco plants. The putative hydroxylase was associated with the leaves and not the entire plant (shoots and roots); however, metabolites of 2,4-DCP were detected in root extracts. Other difficulties with transgenic plants are that they acquire developmental abnormalities such as aberrant shoots, and the expression and regulation of the transferred genes are either incomplete or unstable. Additional research is needed in the area of developing cloned cells to establish their usefulness in plant cell reactors or whole plants for degrading/removing xenobiotics in liquid wastes or soils.

Summary and Future Research Needs

The biodegradation of hazardous waste is currently an exciting field of research, both in terms of microbiology and remediation. New and different organisms are being discovered and studied, some with unusual biochemical mechanisms. Different organisms are being studied for application to the field of bioremediation. This conference exemplified the nature of this field. Very unusual organisms such as those that can dechlorinate PCB were discussed. Molecular biologists discussed the bioengineering of plants for uptake and metabolism of toxic chemicals. The white rot fungi were discussed both in terms of their unusual enzyme system and their ability to mineralize a wide variety of chemicals. The application of biodegradation systems was discussed, but the subject was not a major emphasis in this conference because this conference stressed the research supported by the National Institute of Environmental Health Sciences Superfund Basic Research Program.

Although the conference stressed the basic sciences aspect of the subject, many disciplines must be represented for successful and safe application of bioremediation. Obviously, biodegradation systems must be engineered in such a way as to be effective and economical. It is frequently noted that bioremediation should have an economic advantage. However, there are few data available to document this assumption as the systems have not

been scaled-up, tested, and evaluated. Two major problems exist. First, much work needs to be done on the effectiveness of the biodegradation systems. In many cases questions still remain as to the standards that must be met. For example, it is not clear if bioremediation can meet the standards of 99.99% or 99.999% removal. In other cases it may well depend on action levels that would have to be met. Secondly, the bioremediation systems will have to be shown to be safe. That is, the process, the intermediates, and the products must be shown to have no toxicological or otherwise detrimental effects, or be controlled in such a way that the final product (site) must be rendered nontoxic and safe. These considerations will necessitate much research on biochemical pathways, intermediates, and products, as well as the toxicity or other hazards of these. This research can logically be placed under the auspices of the National Institute of Environmental Health Sciences and the Superfund Basic Research Program.

The data presented on the use of gasoline hydrocarbons by isolated bacterial strains and sediments suggests the existence of a significant microbial diversity in these habitats and the potential for using bioremediation as a treatment technology for contaminated soils and groundwater. The rapid degradation of BTEX under natural groundwater flow and dissolved oxygen conditions (aerobic) has been demonstrated at two well-studied aquifers by workers at the University of Waterloo (*5*) and Rice University and EPA (*6*). These are a few of the definitive studies showing that soil microbes associated with saturated zones can degrade aromatic hydrocarbons and control plume migration under aerobic conditions in the field. Although laboratory soil microcosms and enrichment cultures show that BTEX can be degraded and/or co-metabolized under denitrifying conditions (*7–9*), there have been few published field remediations demonstrating that these compounds degrade under stimulated denitrifying conditions in an aquifer. Preliminary findings at the Bordon aquifer site (*10*) of two gasoline plumes, one undergoing natural aerobic degradation and the other remediated with NO_3^- indicated that the decay of BTEX was similar in both (*10*). Recent findings of a NO_3^- infiltration study at the Traverse City site aquifer indicated that toluene, ethylbenzene, and *m*-xylene, but not benzene or *o*-xylene were degraded under stimulated denitrification. At this time there appear to be some technical uncertainties for implementing field scale remediations using NO_3^-. Some potential problems include *a*)inhibition of complete denitrification by dissolved oxygen levels in natural aquifer plumes; *b*) high variability in the vertical and horizontal distribution of denitrifying microbes in saturated soils; and *c*) the induction of aromatic hydrocarbon degradation under nitrate-reducing conditions may be under critical regulatory constraints due to oxygen, available electron donor, and existing soil redox potentials. Additional studies are required in these areas before field-scale implementation of stimulated denitrification for biodegrading petroleum hydrocarbons is a widely accepted remediation option.

A significant literature basis exists for extrapolation of biologically mediated degradation of chlorinated aliphatic hydrocarbons. However, good evaluative data for application in the field are lacking. The few data available either are years in development and therefore contribute to extended environmental problems (and a deemphasis of the utility of bioremediation) or are not conducted with significant control data to allow academic and government endorsement. The data presented here enhance our understanding and confidence in bioremedial techniques, but much more definitive data are needed for extrapolation. NIEHS could play a significant role in furthering this technology and reducing health and environmental threat by funding areas of both laboratory and field experiments to develop better data on the limits of biodegradation, the extent of conversion in field tests, and the fate of microorganisms in enhanced systems.

In addition, it appears from the volume of information generated in such a short time that there may be other organisms with unique capabilities for metabolism/transformation of persistent environmental pollutants. Data suggest that unique functions may be found at hazardous waste sites if investigated in depth in both field and laboratory studies. This type of work as well as field demonstrations are best implemented by cooperative academic, government, and industry scientists and engineers.

Key research efforts over the next 3 years suggested by Unterman will include: optimization of native type I and type II aerobic PCB-degrading strains (growth and activity for mixed culture applications); genetic engineering of superior aerobic PCB-degraders; enrichment, characterization, and optimization of anaerobic dechlorinaturing cultures; development of application technologies for biotreatment of soils and sediments, i.e., soil slurry bioreactors, *in situ*, two-step anaerobic/aerobic processes, and mixed systems (physical/biological and chemical/biological).

Many studies have shown that microbes can be isolated from soils, sludges, or wastes or cloned for degradative capabilities for a wide variety of xenobiotics (BTEX, PCBs, pesticides and PAHs). There is a lack of information, however, on the applicability of these microbial cultures in pilot or full-scale field tests for enhanced bioremediation of hazardous wastes. Verification of the limits, extent of degradation and cost-effectiveness of biotechnologies when compared to proven remediation methods such as thermal treatment, chemical oxidation, or fixation have not been established. Other aspects of bioremediation technologies that require further research include *a*) determination of the extent of microbial degradation, transformation (metabolites formed), or mineralization (conversion of organic carbon to CO_2) of xenobiotics in actual wastes, *b*) identification of persistent or nonbiodegradable components of a waste, *c*) development of appropriate toxicity assays (e.g., Ames/mammalian cell mutagenicity, cytogenicity, chromosome aberration, micronucleus formation, inhibition of antibody formation, short-term invertebrate/vertebrate animal or plant tests), which are urgently needed for assessing toxicity reduction after bioremediation. Appropriate short-term batteries of tests as predictors of human health risk exposure and site assessment should be developed and evaluated similar to those described for carcinogen detection (DNA damage, mutation, and chromosome abnormalities). Newer *in vitro* assays such as those testing for the effects of xenobiotics or their metabolites on mammalian cell cytotoxicity/cytogenicity (*11*), lymphocyte clastogenicity (*12*), T-lymphocyte antibody formation effects (*13*), or the numerous *in vitro* model bioassays (neurotoxicity, hormone antagonists, heptocyte, skin, and embryonic cell toxicities) currently under development at the Center for Alternatives to Animal Testing at

Johns Hopkins University (*14*) should be evaluated for hazardous waste reduction assessment.

The engineering implementation of biotechnologies for remediating hazardous wastes such as large-scale cultivation and field application of naturally occurring degraders at sites requires additional research. In this respect, molecular biology, genetic engineering, and gene probe technology have advanced beyond our knowledge of how to introduce, maintain, and sustain the growth of introduced microbes at hazardous waste sites. Finally, the government regulatory community, academe, and industry need to interact more effectively to identify biotechnology research needs on waste types, appropriate criteria for waste reduction, biotoxicity and risk assessment of wastes and engineering requirements, and selection of most suitable cost-effective remediation options.

Appendix

Biodegradation of Hazardous Wastes: Program

Session I
Chairperson: A. Bourquin, ECOVA
From biodegradation to organic synthesis with biosystems: genetics, enzymology and bioreactors
B. Witholt, University of Groningen, The Netherlands
Bioremediation of hazardous wastes
R. Unterman, Envirogen, Inc.
Mineralization of pyrene, benzo[a]pyrene, and carbazole by soil microbiota
J. R. Vestal, University of Cincinnati
TCE destruction during growing of *Pseudomonas* on branched-chain, alkyl-substituted benzenes
G. D. Hegeman, Indiana University
Halogenated hydrocarbon degradation by methane-utilizing bacteria
R. S. Hanson, University of Minnesota

Session II
Chairperson: J. Loper, University of Cincinnati
Biodegradation of azo dyes in biofilms
P. Bishop, University of Cincinnati
Aerobic microbial degradation of azo dyes and related dye stuffs
M. W. Tabor, University of Cincinnati
In situ bioremediation of volatile organic compounds
M. Nelson, ECOVA
Aerobic and anoxic BTEX degradation by pure cultures of bacteria isolated from contaminated aquifers
R. Olsen, University of Michigan
Assessment of toxicity in biological treatment of chloro-organic-contaminated groundwater
J. E. Ongerth, University of Washington

Session III
Chairperson: J. Tiedje, Michigan State University
Anaerobic metabolism of toxic priority pollutants
L. Young, New York University Medical Center
Advances in reductive dechlorination
J. Tiedje, Michigan State University
Anaerobic microbial dechlorination of PCB
D. Abramowicz, General Electric Corp.
Metabolism of halogenated phenols in plants
M. P. Gordon, University of Washington
Biodegradation of hazardous wastes by white rot fungi
S. D. Aust, Utah State University
Enzymology of xenobiotic degradation by *Phanerochaete crysosporium*
J. A. Bumpus, Utah State University

Session IV
Chairperson: J. Salanitro, Shell Development Company
Characterization of the heme active site of lignin peroxidase
M. Tien, Pennsylvania State University
Enzyme-catalyzed binding of pollutants to humic material
J. M. Bollag, Pennsylvania State University
Molecular basis of biodegradation of synthetic chlorinated compounds
A. M. Chakrabarty, University of Illinois Medical Center

Session V: Panel Discussion
Chairperson: S. D. Aust, Utah State University
Panel members: W. A. Suk, NIEHS, M. McFarland, Utah State University, R. Colwell, University of Maryland, S. Safe, Texas A&M University, and J. B. Cummings, EPA

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